Role of Fiber Length on Alveolar Macrophage Phagocytosis and Inflammatory Response

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INTRODUCTION

There are over six different commercial classes of the elongated mineral fiber commonly referred to as asbestos and numerous non-commercial types [1]. Collectively, these asbestos fibers are known to induce lung diseases such as asbestosis, mesothelioma, and lung cancer, but little has been done to isolate the immune system's biological response at the cellular level [2]. Despite regulations to limit importation and use of asbestos in the U.S., there have been approximately 100,000-140,000 lung cancer deaths per year worldwide and an estimated 5% to 7% of all lung cancers may be traced back to asbestos. The risks of asbestos-induced pulmonary diseases continue to be a major concern due to the latency between exposure and development of related diseases [3].

The immune system defends itself through a mechanism called phagocytosis during which macrophages, a type of white blood cells, engulf and digest foreign particles and pathogens in the body, such as asbestos. Macrophages will secrete "messenger" proteins, known as cytokines, which signal between cells to stimulate immune responses, along with other proteins and oxygen radicals in an attempt to absorb or break down these particles. Asbestos has been shown to induce the secretion of these extracellular substances, suggesting that cytokines released by pulmonary macrophages may be indicative of biological responses that correlate to the lung inflammation and fibrosis associated with asbestos and other such fibers [4]. This raises many questions concerning the underlying mechanisms that determine the toxicity of asbestos. Some fibers, such as the amphibole fiber, are generally more toxic, possibly as a result of structural factors including length, diameter, and aspect ratio [3]. In particular, one specific discussion at a 2011 workshop on the current risk and research of asbestos suggested that shorter asbestos fibers (< 5μm) do not play a role in inducing disease [1].

Studies of the mechanics of phagocytosis have explored the relationship between size and internalization of spherical particles [5]. However, size is only a major factor when the volume of the particle exceeds the volume of the macrophage and these studies overlooked particle shapes that deviated from spheres, as fibers do [6]. In 2008, Champion and Mitragotri found that elongated polymer particles with high aspect ratios (> 20) were internalized less often than spheres and typically resulted in actin structures only spreading around the particle. Only when the pointed ends of the particle were oriented with the membrane of the macrophage could phagocytosis initialize [7]. Unfortunately, the methods they developed to stretch particles always led to a decrease in diameter as length increased, so it could not be determined whether length or diameter played a larger role. In 2007, a new dielectrophoresis process was developed that allows for the separation of fibers of the same diameter and different lengths [2]. The fabrication and separation of these fibers enable specific investigation of the role in fiber length on phagocytosis.

The objective of this research project was to vary the length of a fiber and observe the effect on phagocytosis by macrophages and on associated immune activities. The interactions of

murine alveolar (lung) macrophages with glass fibers were focused on the internalization of the fibers as well as the presence of inflammatory factors released during the immune response. Specifically, the presence of interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-a) cytokines and reactive oxygen species (ROS) served as biological markers for immune defense and inflammation. We hypothesized that shorter fibers will be internalized by macrophages and produce fewer inflammatory biological responses as depicted below in Figure 1. The data reported below supports this hypothesis though it is not completely clear yet whether for a given dose of fibers, the increase in inflammatory mediators seen for long fibers is merely proportional to the length or if the long fibers are indeed more inflammatory when normalized for length.

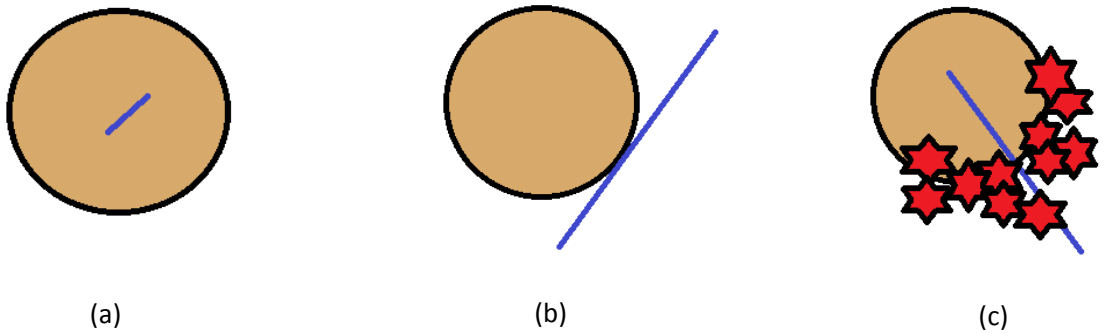


Fig. 1: Three potential fiber-cell interactions hypothesized are shown: (a) glass fiber is readily internalized by the macrophage, (b) Macrophage attaches to the side of a fiber and actin structure cannot orient itself for phagocytosis, or (c) partial phagocytosis occurs and macrophage releases inflammatory factors (IL-1, TNF-a, & ROS).

MATERIALS AND METHODS

Cells

The continuous MH-S murine alveolar macrophage cell line (American Type Culture Collection [ATCC], Manassas, VA, USA) was used due to the cells' life longevity and ability to secrete the IL-1α cytokine involved with inflammatory responses in the immune system. Cells were cultured in RPMI-1640 medium (ATCC) supplemented with 20mM 2-mercaptoethanol, 10% fetal bovine serum, and 1% penicillin/streptomycin and incubated under standard culture conditions (37°C, 5% CO_2, humidified) in cell culture flasks.

Fibers

Glass fibers for the study, modeling asbestos, were obtained from collaborator, Dr. Leonid Turkevich of the National Institute for Occupation Safety and Health (NIOSH). The starting fiber material had a long-normal distribution with a median length of approximately 40µm, 10% less than 10µm, and 10% greater than 130µm. All diameters fell between 0.25µm and 0.75µm. The fibers were then separated by a dielectrophoresis process into two batches,

short fibers with lengths less than or equal to 15µm and long fibers with lengths greater than 15µm.

Time-lapse Video Microscopy

Approximately 12,000 cells in 2mL media were added to a glass bottom microscopy dish and placed in an incubation stage on an Axio Observer.Z1 inverted microscope (Carl Zeiss, Inc.) and examined at DIC 100x magnification. Fibers were added to the center of the dish and images were captured every 2 min by a Zeiss AxioCam camera. Images were collected and compiled into videos using AxioVision software and manually analyzed for cell-fiber interactions. Criteria for determining if phagocytosis: fiber should be in the same focal plane as the cell, never cross over the nucleus of the cell, and remain in the same relative position inside the cell. Examples of time-lapse images are shown in Figure 2.

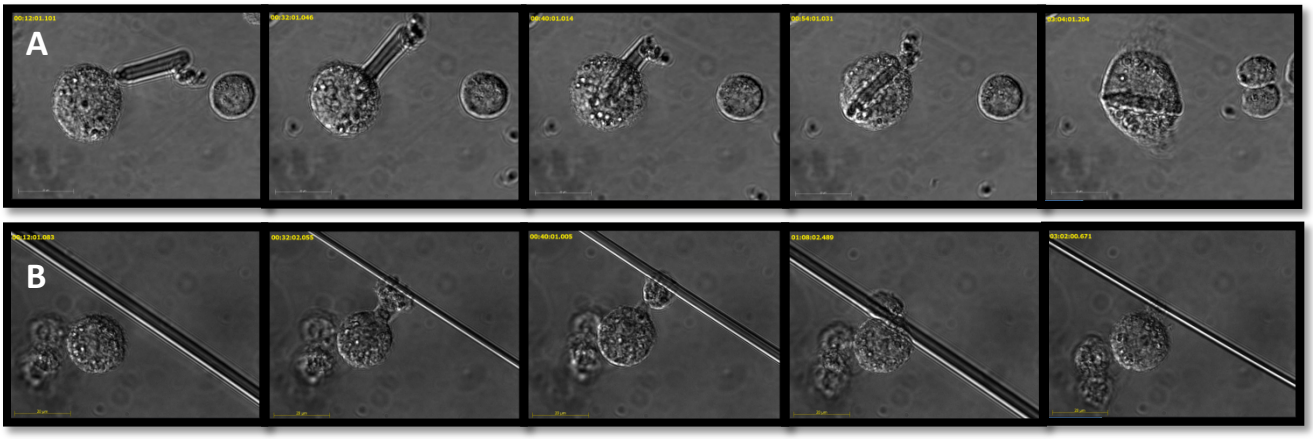


Fig. 2: Time-lapse images of (A) internalization of a fiber and (B) attachment to fiber with no internalization. Image time points depicted above are approximately 12 min, 32 min, 40 min, 60 min, and 120 min after imaging began immediately after the fibers were added to the cell dish.

Fiber Labeling for Fluorescent Studies

Approximately 1.5mg of glass fibers were suspended in 1mL of 1M KOH by sonication (pulse 4sec on/2sec off at 30% amplitude for 2 minutes) and left to soak for one hour. They were rinsed with 1mL MQ water followed by a rinse with 1mL ethanol. Each rinse consists of centrifuging the fibers at 1000rpm for 5min., 5000rpm for 10min, and 10,000rpm for 1 min to maximize fiber retention when removing the supernatant with a pipette while minimizing breakage of longer fibers. After the washes, the fibers were dried in a non-humidified incubator 37°C for approximately 1 ½ hours or until complete evaporation of liquids from fibers. The dry, washed fibers were then incubated for 2 minutes in a solution of 1mL toluene and 33µL 3-mercaptopropyl trimethoxysilane, rinsed with excess toluene to remove unconjugated silane molecules, and suspended in 1mL of a 20mM TES sodium salt solution. 15µm of a 20mM solution of 5-iodoacetamidofluorescein (5-IAF) in dimethyl formamide (DMF) were slowly added to the TES sample while mixing on a stir plate. The fiber solution was covered with

aluminum foil and left to react in the dark for 2 hours at 4°C. The fibers were centrifuged once more and resuspended in 1mL PBS and stored in the dark. Figure 3 displays flow cytometry data verifying the fluorescent labeling of the glass fibers.

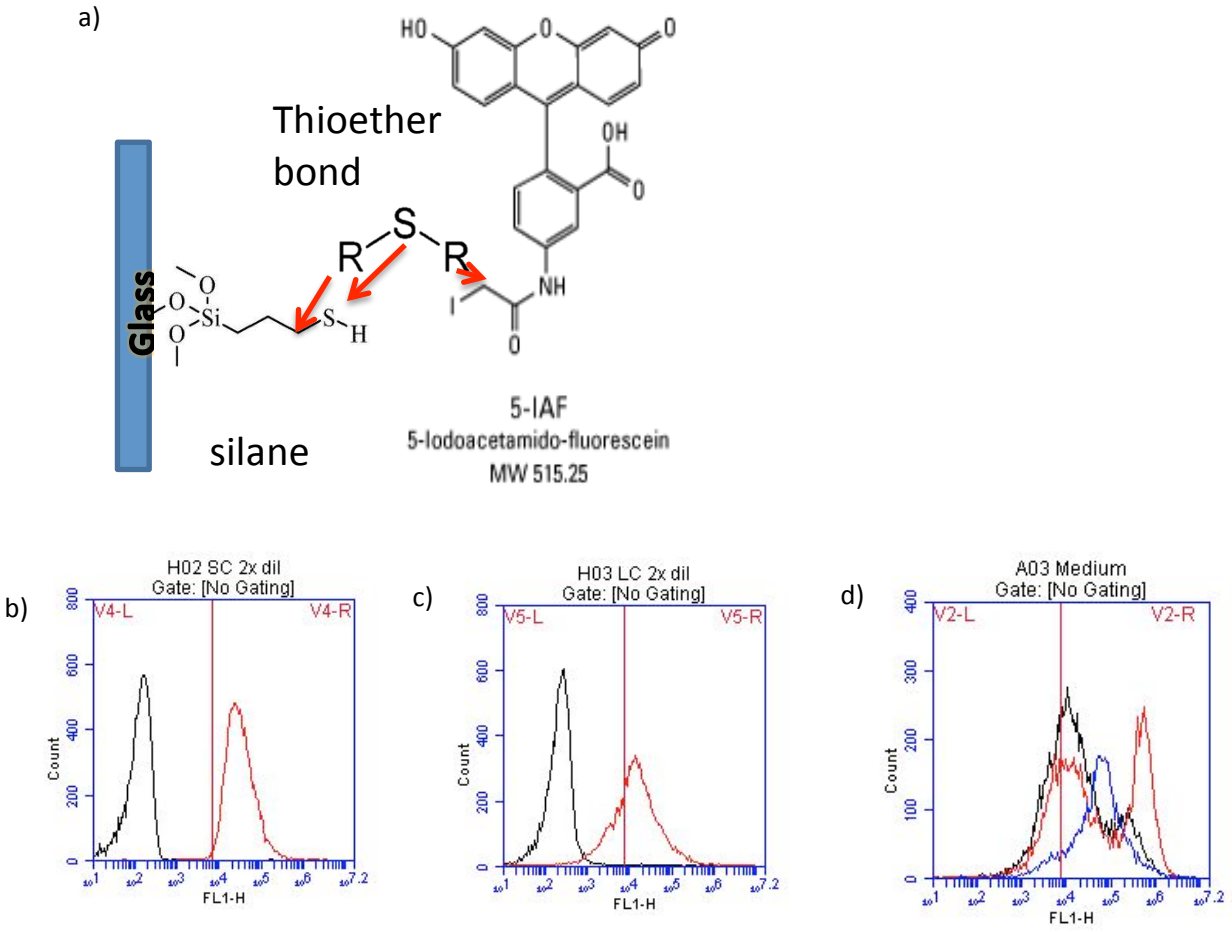


Fig. 3: Characterization of fluorescent fibers. (a) Schematic showing conjugation chemistry. (b-d) Histograms show the fluorescent distribution of fibers. The red vertical gate marks the upper limit of the autofluorescence of the cells. (a) Short fibers unlabeled (black) and labeled (red). (b) Long fibers unlabeled (black) and labeled (red). (c) Long fibers labeled with varying quantities of silane and 5-IAF. The blue peak represents fibers that were filtered prior to labeling to narrow the distribution and labeled using 15µL silane and 10µL 5-IAF. When the fibers underwent the pre-filtration, 89.58% of the 5,000 events measured were above the fluorescence level of cells.

Flow Cytometry

Thirty-six wells of a 48 well plate were filled with 0.5mL media solution at a cell concentration of 6.67 x 10^4 cells per well and incubated for 24 hours. Three different concentrations each of both short and long fibers were added with 0.5mL new media to make six wells of each different concentration combination. Two wells of each concentration were assayed after incubation periods of 6, 12, and 24 hours. At each time point, cells were scraped out of wells, centrifuged at 1000 rpm for 5 min., washed with 500µL PBS, centrifuged following the same progression used when labeling the fibers, and resuspended in 200µL PBS. Samples

were then filtered through a 35μm mesh and assayed on the BD Accuri C6 flow cytometer using BD Accuri C6 software set to collect 10,000 events at medium speed (results shown in Figure 5). Cells analyzed in this way only measured "association" of fibers with cells, and could not distinguish between fibers that were inside cells and those that were outside cells.

To distinguish between fibers that were inside versus outside, cells exposed to fibers were trypsinized to remove fibers attached to the exterior surface of cells but not internalized. The trypsin enzyme cleaves receptors on the cell surface, including those bound to fibers. However, in a control experiment in which cells were exposed to fibers at 4ºC, where they could not be internalized, trypsin failed to remove bound fibers as demonstrated by no significant difference between trypsinized and un-trysinized cells. An alternative method was explored to differentiate between internalized and attached fibers, instead using trypan blue to quench the fluorescence of attached fibers. Eight wells of a 48-well plate were filled at a concentration of 5.0×10^4 cells per well, the same concentration used for ELISA experiments below, and incubated for 24 hours at 37ºC. Media was replaced with fresh solution containing short fibers in concentrations of 5, 10, and 20 fibers per cell with two wells of each concentration and incubated for an additional 24 hours. Cells and fibers were scraped from each well and centrifuged, and the culture media was removed. The samples were resuspended in 200μL of PBS, and 100μL of each sample was mixed with and additional 100μL PBS, filtered, and run through the flow cytometer at medium speed until 10,000 events were captured. The other 100μL of each sample was mixed with 100μL of trypan blue and assayed under the same parameters. Results were gated to exclude free fibers, and the percentage of cells that fluoresced above cells' autofluorescence after trypan blue quenching was subtracted from the relative fluorescence of cells only suspended in PBS. This difference was attributed to attached fibers that were quenched by the addition of trypan blue.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were added to 24 wells of a 48 well plate at a concentration of 4×10^4 cells per well and incubated for 24 hours. Three samples were left alone as negative controls, three were given *E. coli* lipopolysaccharides (LPS) at a concentration of 1μg/mL, three were given long fibers at a concentration of 5 fibers per cell, and three were given short fibers at the same concentration of 5 fibers per cell. The samples were incubated for another 24 hours. An ELISA for the mouse IL-1α cytokine was performed using R&D Systems DuoSet ELISA Development kit. The enclosed protocol was used to complete the assay with all solutions and materials diluted to R&D specifications. To prepare ELISA samples, supernatants were removed from the cells and centrifuged to remove debris and placed in duplicate in Nunc 96-well plates. Seven standards of two-fold serial dilutions were made as specified in the protocol and a final sample of only reagent diluent was used. The absorbance of each sample was read on a plate reader at 410nm, 490nm, and 540nm. A logarithmic seven-point calibration curve was determined using the seven standards read at 410nm and corrected by subtraction of absorbance values read at 540nm. This general procedure was repeated for a variety of fiber doses and for TNF-a ELISA measurements.

Reactive Oxygen Species (ROS)

Cells were plated and incubated with fibers in the same manner as for ELISA. In an effort to optimize labeling of ROS, a variety of conditions were tried using two different ROS

dyes from Invitrogen, CellROX green and CellROX far red. In addition to their fluorescent signal, the mode of ROS labeling is different and their background fluorescence. In the literature CellROX far red is preferred because its spectrum does not overlap with cell autofluorescence, it is non-fluorescent when not oxidized by ROS, and it labels ROS in the cytoplasm of the cell. Green does overlap, has low fluorescence when not oxidized, and labels in the nucleus and mitrochondria. To establish the positive control, 0.5-1µg/mL LPS was added to cells for 1-24 hours. 5-20µM CellROX green or red was added either directly to the cell media, or the media was removed and the dye was added to the cells in PBS. Cells with incubated with dye for 30 minutes at 37ºC. In some cases Hoechst nuclear stain was added for the last 15 minutes to be able to count live cells in the sample for normalization. Cells were either scraped from the wells, washed 3x in PBS and analyzed by flow cytometry, or washed 3x in their wells and analyzed by florimetry (ex/em is 488/520 for green, 640/665 for red).

RESULTS and DISCUSSION

Video Microscopy

Twenty-one total videos were collected depicting a combined 25 events (Figure 4), where an event is defined as a visual interaction between a cell and fiber whether it be attachment to or internalization of the fiber. Video microscopy has been found to be a useful visual aid in monitoring the behavior of the macrophages. Categorization as a "long" or "short" fiber was determined by measuring the fibers' length within the AxioVision software. Long fibers are those greater than 15µm in length, and short fibers are those less than or equal to 15µm. 25% of cell attachments to short fibers resulted in phagocytic internalization, but only 12% of long fiber attachments resulted in internalization. It is evident that most attachments to long fibers will not result in internalization. This is mostly likely due to an inability of the macrophage to wrap around the entire length of longer fibers. When phagocytosis was observed, it was also noted that the fiber was typically oriented perpendicular to the surface of the cell (attached at its pointed end) as was found in previous particle studies with rod-shaped particles.

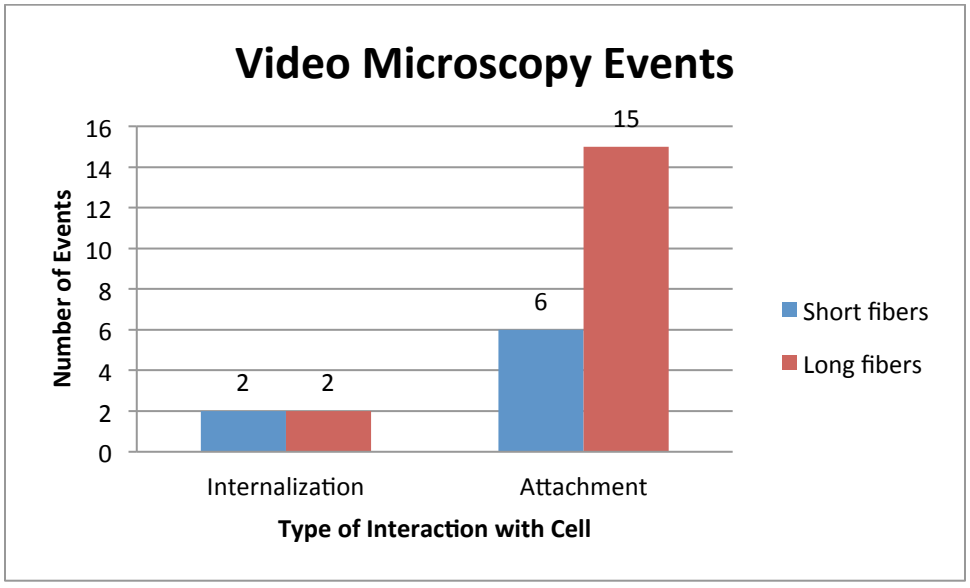
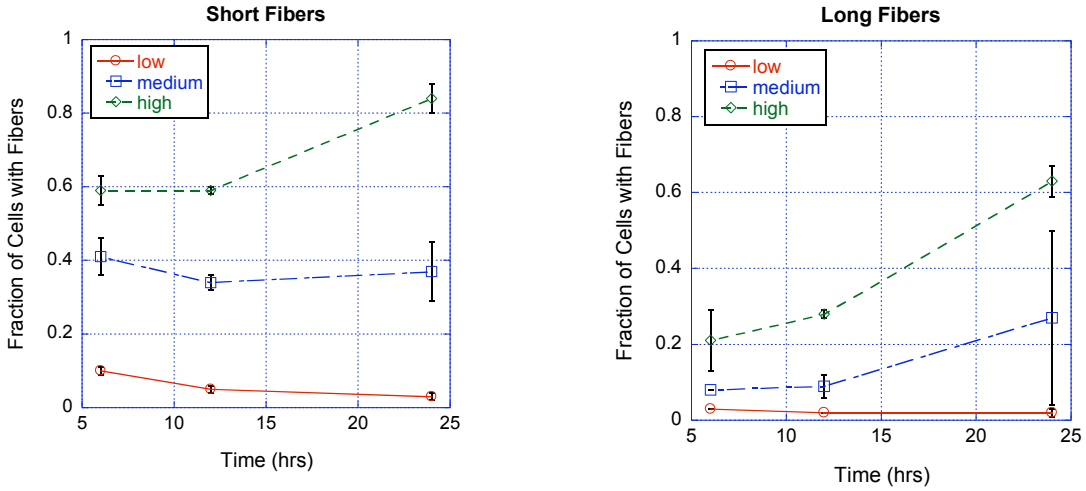


Fig. 4: Bar graph showing interactions observed manually in time-lapse videos when short and long fibers were introduced to a cellular environment.



	Fibers per Cell		
	low	med	high
Short	0.20	19.73	39.45
Long	0.02	0.10	0.19
Short/Long	8.26	206.58	206.58

Fig. 5: Line graphs show the fraction of cells for each sample that internalized or attached to a fiber of the specified length set. The table lists the actual concentrations of fibers (in fibers/cell) added to each sample. For the two highest concentration levels, there were 200 times as many short fibers given to each cell as long fibers.

Flow cytometry data that does not separate attachment from internalization is inconclusive (Figure 5). There does not seem to be a correlation between incubation time and fiber uptake for either long or short fibers, although there did seem to be a large quantity of attached or internalized fibers after 24 hours. Both types of fibers follow a similar trend of more uptake if a higher concentration of fibers is present, but the two data sets cannot be directly compared due to much higher concentrations of small fibers being added to increase the number of interactions.

Quantitative data of cell-fiber interactions shows a strong dose dependence for short fibers (Figure 6). Additionally, the ratio of attached to internalized short fibers is in agreement with the timelapse movies. Due to clogging of the flow cell, these studies have not yet been performed for long fibers. When the issue is resolved, uptake and attachment of long fibers will be assessed for low doses. Long fibers tangle at concentrations above ~2 fibers/cell.

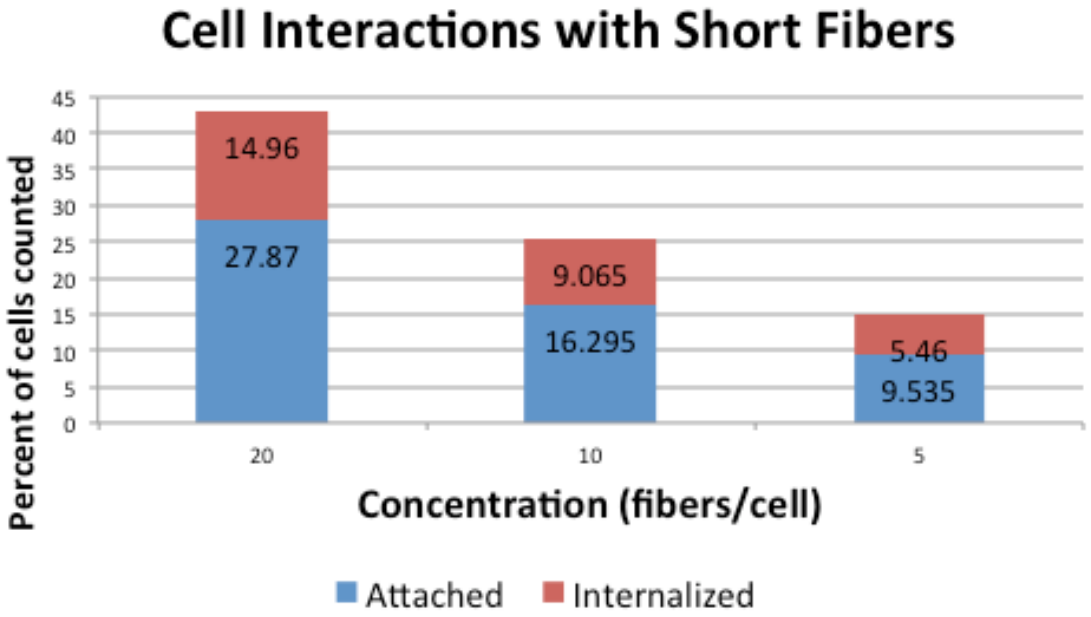


Fig. 6: Percentages of cell fluorescence due to different cell-fiber interactions. Overall, higher concentrations of short fibers had relatively greater number of interactions.

ELISA

A variety of fiber doses were tested. All ELISA data for IL-1 fell below the stated limit of the assay. However, the samples were non-zero according to the calibration curve and were significantly different from the "no treatment" controls. As an alternative, since production of TNF-a is higher in MH-S cells than IL-1, TNF-a production was also assessed. These levels were indeed higher compared to IL-1 (Figure 7). In the dose assayed, IL-1 appeared to have a nearly linear relationship with dose and the slope of the long fiber curve was ~10x the slope of the short fiber curve. This hints at an inflammatory effect of long fibers beyond the fact that they are simply ~5-10x bigger than short fibers. TNF-a, on the other hand, appeared to have a more complex shape with highest slopes in the mid-dose range.

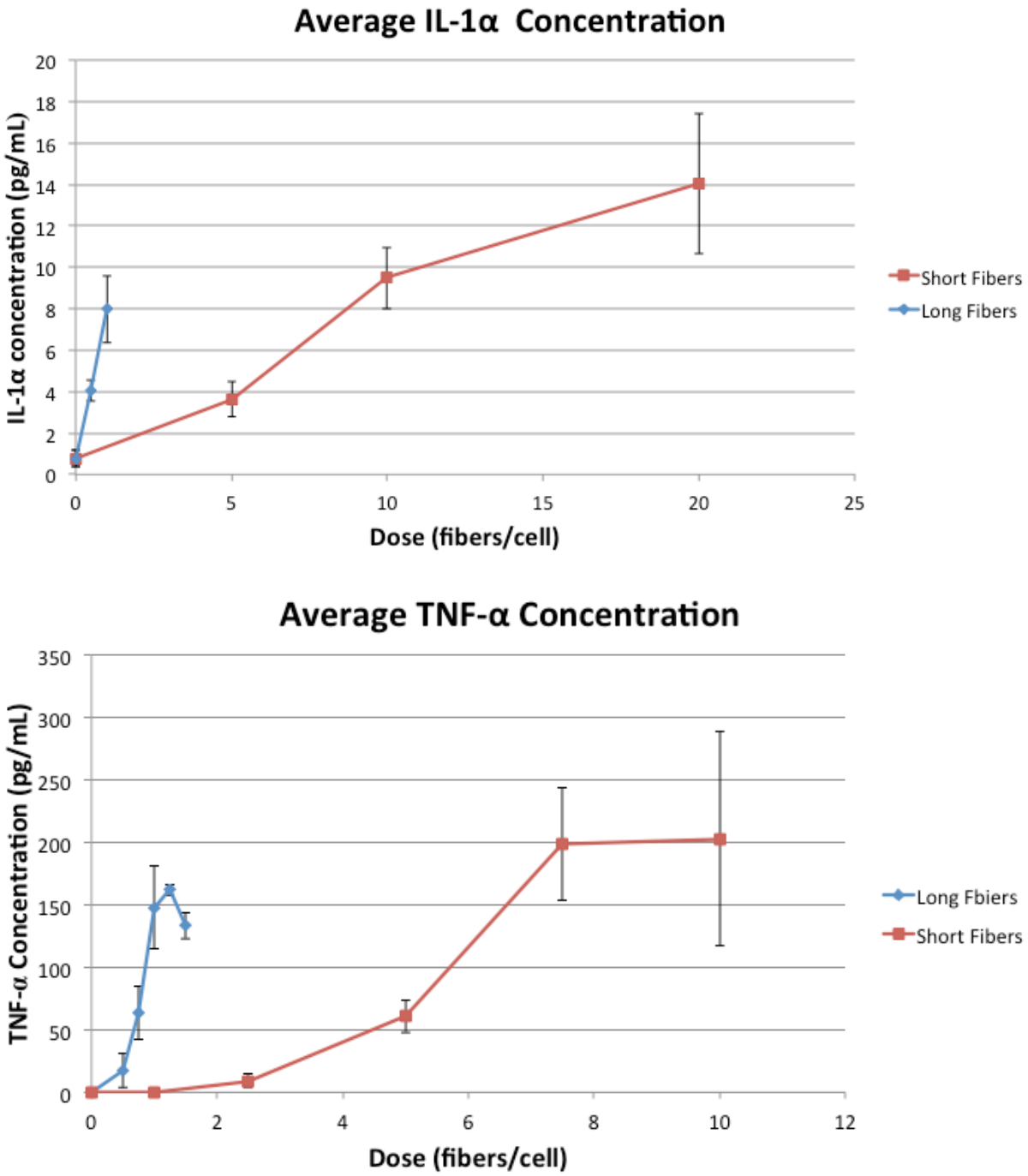


Fig. 7: These dose response curves for IL-1 and TNF-a production in response to fibers are representative of data collected. Error bars represent the standard deviation between the two concentrations read for each type.

ROS

Unfortunately, as yet, we have not been able to establish the ROS assay with our positive control, LPS. Complicating factors are loss of cells due to LPS exposure and presence of

reducing agents in cell culture media. We are still working on this and have more experiments planned.

CONCLUSIONS

In summary, we demonstrated that long and short fibers interact with cells differently and in a dose dependent fashion. Long fibers exhibited less uptake than short fibers, but elicited significantly higher production of inflammatory cytokines IL-1 and TNF-a. These results will be completed this summer and submitted as a manuscript co-authored by Georgia Tech and NIOSH researchers. This information will be a significant contribution towards understanding the molecular interactions between fibers and immune cells that contribute towards toxicity. There are a variety of other measurements and questions that can be asked, to address the mechanism of fiber toxicity with single cells. If interest from NIOSH remains high, we are happy to continue to work together on this important public health issue.

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